Regulation of the Receptor for Advanced Glycation End Products by Estrogen Receptor Ligands in Endometrial Cancer

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Introduction

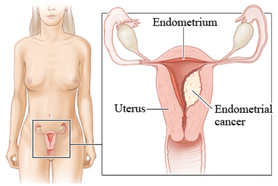

Figure 1. The uterus and endometrial cancer

Endometrial cancer (EC) is the most common gynaecological malignancy in western countries with established risk factors such as excessive unopposed estrogen exposure and the use of tamoxifen to treat ER+ breast cancer. Such risk factors may promote inflammation, an important hallmark in cancer. Altered 17β estradiol (E_2) ER isoform and co-regulator levels are implicated in some cancers including EC. An increased risk of EC has been observed in a subset of patients receiving the drug tamoxifen (TX), an anti-estrogen to treat ER+ breast cancer. The partial agonist behaviour of TX observed in the endometrium is thought to occur due to the different cellular and gene promoter context, as well as the involvement of specific ER isoforms and ER co-regulators enhancing the expression of some target genes that may drive cancer development in the endometrium. To date, it is unclear which patients are at risk of the adverse effects of TX in the endometrium, therefore a biomarker to predict and assess this risk is needed. The receptor for advanced glycation end products (RAGE) is a single trans-membrane member of the immunoglobulin family involved in several pathways including inflammation, apoptosis, proliferation and cancer. RAGE has been shown to be elevated in a number of pathologies linked with inflammation and has been shown to be over expressed in many cancer types including prostate, breast and pancreatic. Tanaka *et al.* has previously shown ER ligands up-regulate RAGE expression in the endothelium, an estrogen responsive tissue via ERα/Sp-1 complexes. As the endometrium is an estrogen responsive tissue, estrogen may modulate RAGE expression in this tissue via the ER. This work aims to elucidate the potential of RAGE as a biomarker in EC and whether it can be used to predict the effect of TX in the endometrium of breast cancer patients. This work has also investigated the molecular mechanisms that regulate RAGE expression in EC following ER ligand exposure *in vitro*.

Materials and methods

Immunohistochemistry (IHC) - samples were fixed in 10% formaldehyde and embedded in paraffin. 3-4µm sections were cut, de-paraffinised and fixed on coated slides. Sections were labelled with anti-ERα or anti-RAGE antibodies and the presence of the protein was identified using a colorimetric detection assay.
Cell culture and treatments – Four endometrial cancer cell lines; Ishikawa (type I), HEC-50, HEC-1A and HEC-1B (type II) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 supplemented with 10% Foetal Bovine Serum (FBS), glutamine 1.5mM, sodium bicarbonate 1mM, sodium pyruvate 1mM and 1% pen-strep at 37°C in a 5% CO_2 humidified incubator. Cells were seeded into 6 well plates and when 90% confluent, cells were either left untreated or treated with 17β estradiol (E_2) 10nM or 4-hydroxytamoxifen (TX) 10nM for 4 hours.
RNA extraction and qPCR – Following treatment, total RNA was extracted using an RNeasy Mini Kit (Qiagen) and quantified using a Nanodrop ND-1000 Spectrophotometer. RNA was reverse transcribed (Applied Biosystems) and amplified with specific primers. Data was normalised using the reference gene RPL-19.
Western Blot – Total protein was extracted in RIPA buffer and quantified using the DC protein assay (Bio-Rad). Proteins were separated with a 4-15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) and transferred to a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were then blocked overnight. Membranes were incubated with the primary antibodies ERα (sc-543 Santa Cruz) and GAPDH (sc-25778 Santa Cruz). Membranes were incubated with an anti-rabbit secondary antibody linked with horseradish peroxidase and membranes were then visualised with a ChemiDoc imager (Bio-Rad).
Chromatin Immunoprecipitation (ChIP) – ChIP was used to assess recruitment of ERα and co-regulators (SRC-2 and NCoR) to Ap1 and Sp1 sites located in the RAGE promoter following treatment with E_2 (10nM) or TX (10nM) using the ChIP qPCR Protein A kit (Chromatrap® product code: 500071).

Results

RAGE and ERα endometrial expression in a patient cohort by immunohistochemistry

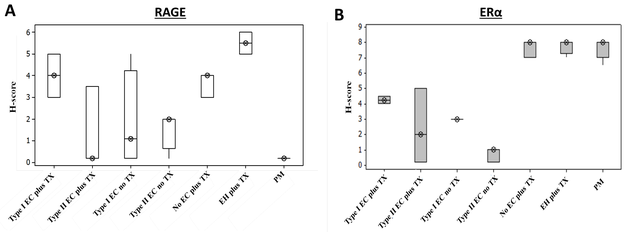

Figure 2. RAGE and ERα endometrial expression in a patient cohort. Box plot show RAGE (A) and ERα (B) endometrial expression in the patients groups enrolled in the study. 138 patients were grouped as follows: PM (n=25), EH plus TX (n=21), no EC plus TX (n=19, type I EC no TX (n=18), type II EC no TX (n=17), type I EC plus TX (n=21) and type II EC plus TX (n=17). IHC samples were scored blind by three independent observers in triplicate. Values shown are median IHC scores and statistical analysis was performed using a Mann-Whitney test.

Protein expression of RAGE in fertile, hyperplasic and tamoxifen treated endometrium

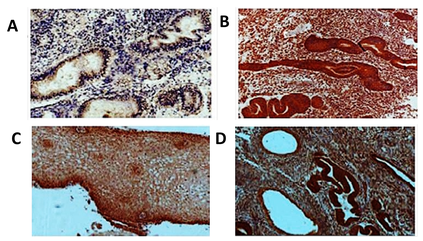

Figure 3. RAGE expression in fertile, hyperplasic and tamoxifen treated patients. Compared to the control fertile patient (A) a patient not receiving hormonal therapy with spontaneous endometrial hyperplasia and progressed to endometrial cancer (B) shows high RAGE expression. A patient receiving TX for breast cancer displaying early hyperplasia at the initial biopsy (C) had elevated RAGE levels compared to the fertile control. A second biopsy was taken from the same patient 3 months later and displayed late stage hyperplasia (D) showing higher RAGE levels compared to the first biopsy.

Basal ERα isoform protein expression in model endometrial cancer cell lines

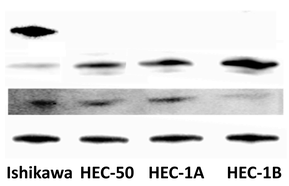

Figure 4. Basal expression of ERα isoforms in Ishikawa, HEC-50, HEC-1A and HEC-1B cells. Immunoblots show each of the endometrial cell lines express differential expression of the ER isoforms.

ER ligands can modulate RAGE mRNA endometrial expression

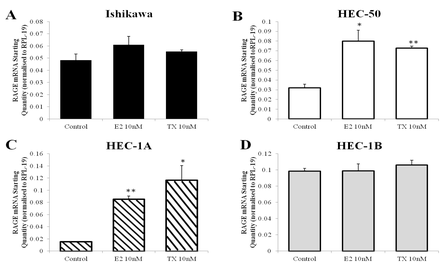

Figure 5. RAGE mRNA expression following E_2 and TX exposure in endometrial cancer cell lines. Results show that target mRNA is induced by both E_2 and TX treatments in HEC-1A cells No effect was observed on target transcript in HEC-1B cells following E_2 and TX treatment.

ERα binding to the RAGE promoter following ER ligand exposure

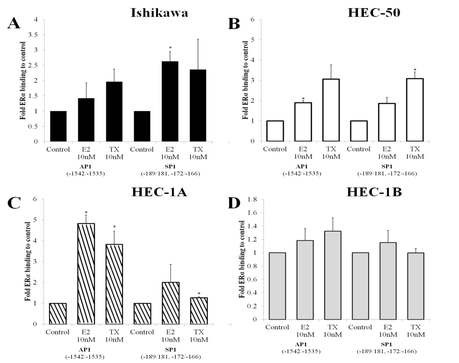

Figure 6. ERα binding to AP-1 and Sp-1 sites in the RAGE promoter following ER ligand treatment in Ishikawa (A), HEC-50 (B), HEC-1A (C) and HEC-1B endometrial cells (D). By ChIP, ER ligand treatment is shown to alter the recruitment of ERα to the RAGE promoter in the endometrial cancer cell lines.

ER ligands alter the co-activator SRC-2 binding to the RAGE promoter

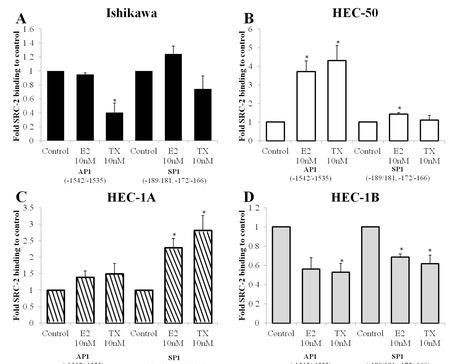

Figure 7. The effect of ER ligand exposure to the recruitment of SRC-2 to AP-1 and Sp-1 sites in the RAGE promoter in Ishikawa (A), HEC-50 (B), HEC-1A (C) and HEC-1B cells (D). By ChIP, ER ligand treatment increases the recruitment of SRC-2 to the RAGE promoter in HEC-50 and HEC-1A cells however bindin was reduced in the Ishikawa and HEC-1B cell lines.

ER ligands alter the co-repressor NCoR binding to the RAGE promoter

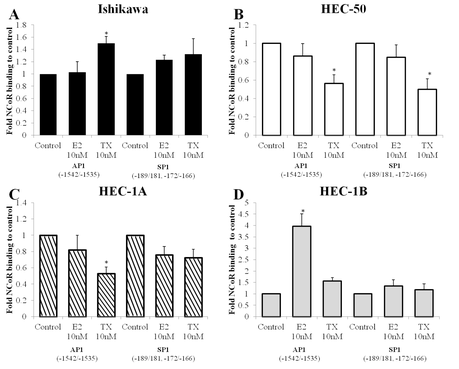

Figure 8. The effect of ER ligand exposure to the recruitment of NCoR to AP-1 and Sp-1 sites in the RAGE promoter in Ishikawa (A), HEC-50 (B), HEC-1A (C) and HEC-1B cells (D). By ChIP, ER ligand treatment decreases the binding of NCoR to the RAGE promoter in HEC-50 and HEC-1A cells however binding was increased in the Ishikawa and HEC-1B cell lines.

ER ligands cause a shift in co-regulator recruitment to the RAGE promoter

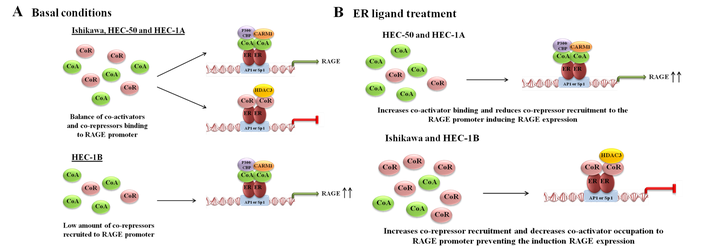

Figure 9. ER ligand exposure induces a switch in co-regulator recruitment to RAGE promoter in endometrial cancer cell lines. In basal Ishikawa, HEC-50 and HEC-1A cells there is a balance of co-regulators and HEC-1B cells show low co-repressor binding at the RAGE promoter. In ER ligand treated cells there is a shift in co-regulator binding. In HEC-50 and HEC-1A cells there is an increase in co-activator and reduction in co-repressor binding and in Ishikawa and HEC-1B cells there is an increase in co-repressor and decrease in co-activator recruitment to the RAGE promoter.

Conclusions

- RAGE and ERα endometrial expression is altered in EC patients and patients receiving tamoxifen compared to postmenopausal women. Therefore RAGE may be a promising biomarker for EC and assessing the agonist action of tamoxifen in the endometrium.
- RAGE is expressed in type I and II EC cell lines and its expression can be modulated by estrogen and tamoxifen in the endometrium.
- By ChIP ERα isoforms and ER co-regulators are recruited to the RAGE promoter to regulate expression of genes in response to estrogen and tamoxifen in the endometrium. This shows the balance of ER isoforms and co-regulators in the endometrium may be important in the expression of RAGE and this may be implicated in the agonistic behaviour of tamoxifen in the endometrium.

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